IN VITRO COVALENT BINDING TO DNA OF THE ULTIMATE CARCINOGEN *N*-ACETOXY-*N*-4-ACETYLAMINOBIPHENYL

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1. Introduction

Recent studies from this laboratory [1] have demonstrated that the in vitro reaction of the ultimate carcinogen *N*-acetoxy-*N*-2-acetylaminophenanthrene (*N*-AcO-AAP) with native DNA induces the destabilization of the double helix as seen by the decrease in melting temperature. It has also been shown that beside the reaction of binding of a 2-acetylaminophenanthrene residue (AAP) there is an acetylation reaction of native and heat denatured DNA. It has been assumed that the acetylation reaction results from the attack of the DNA by the carbocation formed by heterolytic cleavage of the O–Ac bond of *N*-AcO-AAP [2]. On the other hand the binding of an AAP residue results from the attack of the DNA by the nitrenium ion (or a mesomeric form of this electrophilic intermediate) formed by heterolytic cleavage of the N–OAc bond of *N*-AcO-AAP [2,3]. Similar studies have now been carried out with the analogous compound *N*-acetoxy-*N*-4-acetylaminobiphenyl, an ester of the proximate carcinogen *N*-hydroxy-*N*-4-acetylaminobiphenyl which is a potent mammary carcinogen in the rat [4].

By means of a specific double labelling of the *N*-acetoxy group with ^{3}H and of the *N*-acetyl group with ^{14}C, we showed that beside the biphenylation of the DNA bases (i.e., the covalent binding of a *N*-4-acetylaminobiphenyl residue) there is an acetylation reaction of the DNA during its reaction with the ultimate carcinogen. Throughout this paper, the term biphenylation will be used in its broad sense (i.e. irrespective of whether the reaction is in fact a true reaction of arylation or rather a reaction of arylamidation). The amount of biphenylation and of acetylation has been investigated as a function of the DNA secondary structure by comparison of the reactivity of native and heat denatured DNA.

The analysis of the melting profiles of the modified DNA gives evidence for a destabilizing effect.

2. Materials and methods

All chemicals were reagent grade (Merck). Native DNA was prepared from chicken erythrocytes [5] and had the following characteristics: hyperchromicity at 260 nm = 41%, $\epsilon_P^{260} = 6400$. Protein content was lower than 0.3% by weight.

Heat denatured DNA was obtained by heating a buffered solution of native DNA in a sealed vial to 100°C for 10 min. To avoid DNA renaturation, the sample was rapidly chilled in ice. The hyperchromicity at 260 nm of the denatured DNA was equal to 28%, but the melting profile of such a DNA showed no cooperativity.

2.1. N-*Acetoxy*-N-*arylacetamides*

N-Hydroxy-*N*-4-[^{14}C]acetylaminobiphenyl has been synthetised starting from 4-nitrobiphenyl (Fluka) and [^{14}C]acetic anhydride (CEA) by a previously published method [6]. The ^{14}C-labelled hydroxamic acid is then acetylated with acetic anhydride in chloroform, in presence of triethylamine to yield *N*-acetoxy-*N*-4-[^{14}C]acetylaminobiphenyl (*N*-AcO-[^{14}C]AABP). The ultraviolet spectrum showed a maximum at 263 nm with a molar extinction coefficient of $\simeq$ 19 560 in ethanol.

N-[^{3}H]acetoxy-*N*-4-acetylaminobiphenyl (N-[^{3}H]-AcO-AABP) was prepared in the same manner by acetylation with [^{3}H]acetic anhydride of *N*-hydroxy-*N*-4-acetylaminobiphenyl.

N-[^{3}H]acetoxy-*N*-4[^{14}C]acetylaminobiphenyl (*N*-[^{3}H]AcO-*N*-[^{14}C]AABP) was obtained by mixing one volume of a solution of *N*-[^{3}H]AcO-AABP in benzene:ethanol (50:50) (5 mg/ml) with three volumes of a solution of *N*-AcO-[^{14}C]AABP in benzene:ethanol (50:50) (5 mg/ml). The benzene:ethanol (50:50) solutions were stored at −20°C, under an argon atmosphere.

Using Unisolve liquid scintillator (Koch Light Laboratories Ltd, Colnbrook, Bucks, England) the specific activity was equal to 9470 cpm/nm and 150 cpm/nm for *N*-[^{3}H]AcO-AABP and *N*-AcO-[^{14}C]AABP, respectively. The specific activity of the double labelled compound *N*-[^{3}H]AcO-[^{14}C]AABP was equal to 1000 cpm/nm for ^{3}H and to 920 cpm/nm for ^{14}C, respectively.

2.2. *Reaction of* N-*AcO-AABP with DNA*

It appears from kinetic studies [3] that the rate of solvolysis of *N*-[^{14}C]AcO-AABP in various buffers (measured as the rate of formation of watersoluble [^{14}C]acetate ions) is almost the same as that measured for *N*-[^{14}C]AcO-AAP. Therefore, the DNA samples (concentration $\simeq 1.5 \times 10^{-3}$ M nucleotide/litre) were incubated under the same conditions as those determined for *N*-AcO-AAP (i.e. at 50°C during 3 days under an argon atmosphere in the dark in 0.01 M (pH 7) sodium citrate buffer : ethanol (80:20, v/v)). In this buffer, the incubation temperature is 10°C below the melting temperature of the native DNA. When higher amounts of DNA modification were wanted, an additional quantity of *N*-AcO-AABP was added after an initial 24 h incubation period and incubation was continued for an additional 48 h. DNA is subsequently purified by three diethyl ether extractions. Thin-layer chromatography on silica gel (solvent : 95% $CHCl_3$: 5% acetone) was used to check that no native *N*-AcO-AABP is left in the diethyl ether extracts, indicating that the solvolysis reaction was completed under the incubation conditions described above. The remaining non-covalently bound biphenyl residues were removed by extensive dialysis against 2 mM sodium citrate buffer (pH 7).

The amount of biphenylation or acetylation is obtained from the ratio $[C]/[P]$ where $[C]$ is the concentration of [^{14}C]acetylaminobiphenyl ([^{14}C]-AABP) or [^{3}H]acetyl, respectively, determined by counting aliquots of 100 µl DNA solutions (Unisolve liquid scintillator), and $[P]$ the concentration of nucleotides as determined by the absorbance at 260 nm. The absorbance at 260 nm has been corrected for the biphenyl contribution by assuming a molar extinction coefficient of the bound biphenyl equal to that of the free biphenyl, i.e., 18 200 at 260 nm.

The ultraviolet spectra of the AABP modified DNA samples show a slight absorption in the 325–305 nm range. By plotting the ratio A_{305}/A_{257} versus the percentage of biphenylation, one obtains a direct spectrophotometric determination of the amount of AABP residues bound to the DNA. Figure 1 shows this curve in the case of native DNA.

2.3. *Helix–coil transition of native and* N-*AcO-AABP reacted DNA samples*

The melting curves of native and carcinogen reacted DNA were measured at least twice for each sample at 260 nm with an XY recorder indicating absorbance versus temperature [7]. In order to expand the temperature scale and to obtain a better accuracy in

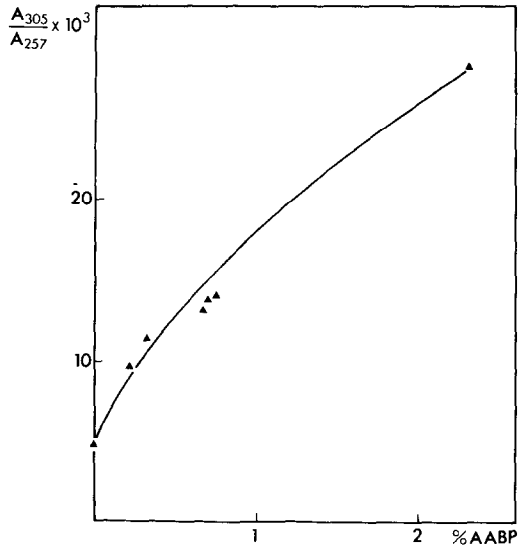

Fig.1. Evolution of the ratio absorbance at 305 nm (A_{305}) over the absorbance at 257 nm (A_{257}) of modified DNA (DNA–AABP) versus the percentage of biphenylation.

Table 1
Amount of biphenylation (% AABP) and acetylation (% acetylation) of native DNA and denatured DNA

R[a]	% AABP[b]		% Acetylation[b]	
	Native DNA	Denatured DNA	Native DNA	Denatured DNA
2	0.32	1.32	0.11	0.73
4	0.75	3.62	0.24	1.75
8	2.31	8.56	0.44	3.26

[a] R = Concentration of the carcinogen/concentration of nucleotides in DNA during the incubation
[b] The amount of biphenylation (% AABP) and acetylation (% acetylation) are obtained by the ratio $[C]/[P]$ where $[C]$ is the concentration of [^{14}C]AABP or [^{3}H]acetyl and $[P]$ the concentration of nucleotides

T_m measurements, we have used as a reference a bath maintained at 43°C ± 0.01°C. All the thermal transitions were performed in 2 mM sodium citrate buffer (pH 7). In this buffer the T_m of our DNA is of 59°C.

3. Results and discussion

3.1. *Reaction of biphenylation*

The reaction of biphenylation as described by the % AABP covalently bound to DNA (see Materials and methods) depends on the secondary structure of DNA, since heat denatured DNA reacts 4–5-times more than native DNA (table 1). This result parallels the reactivity pattern obtained for the *N*-acetoxy-*N*-2-acetylaminofluorence (*N*-AcO-AAF) as first observed by Miller et al. [8] and is in contrast with the finding that *N*-acetoxy-*N*-2-acetylaminophenanthrene (*N*-AcO-AAP) reacts to the same extent with native and heat denatured DNA [1]. However, the extent of native DNA modification obtained with both the biphenyl and the phenanthryl derivative are similar and very low when compared to the exceptionnally high reactivity of the fluorene derivative [3]. Under given conditions, the biphenyl derivative has been found to react 2–3 times more with guanosine than the phenanthryl derivative [3]. Our results with denatured DNA confirm this observation (table 1). However, with native DNA the biphenyl derivative is less reactive than its phenanthryl analog (table 1) reflecting the fact that, curiously, the double helical structure of DNA does not lower the extent of *N*-AcO-AAP binding: this is in contrast with all the other *N*-acetoxy-*N*-arylacetamides we have tested so far, and whose reactivity toward denatured DNA always exceeded that of native DNA.

3.2. *Reaction of acetylation*

The acetylation reaction is followed by the incorporation of the ^{3}H-label into the DNA molecule after its reaction with *N*-[^{3}H]AcO-[^{14}C]AABP (see Materials and methods for the definition of the % acetylation). The acetylation reaction by *N*-AcO-AABP has first been observed by Miller and Miller [9] on guanosine. These authors have shown that the acetylation of guanosine is the primary reaction observed with *N*-AcO-AABP [9].

In this paper we show (table 1) that the acetylation reaction also occurs with native and heat denatured DNA. There is a seven-fold decrease of the acetylation reaction when going from denatured to native DNA (table 1). Such an effect has already been described for the phenanthrene derivative [1]. The sites at which the acetylation takes place have so far not been investigated.

3.3. *Thermal destabilization of* N-*AcO-AABP modified DNA samples*

It is observed that the DNA melting temperature decreases when the extent of reaction with *N*-AcO-AABP increases. In fact, the problem remains to show whether this destabilization is due to the biphenylation or the acetylation reaction (or both). We have no answer to this question, but since in the

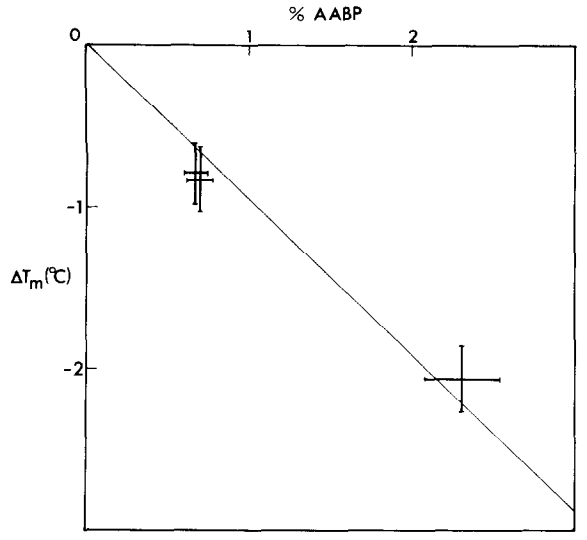

Fig.2. Destabilization ΔT_m (°C) as a function of the percentage of biphenylation.

case of *N*-AcO-AAF [10] and of *N*-AcO-AAP [1] an analogous destabilization has been observed and attributed to the binding of 2-acetylaminofluorene (AAF) and AAP residues respectively, we may reasonably assume the same to be true in the case of *N*-AcO-AABP. If so, the destabilization is equal to 0.95°C per percent bound AABP residue (fig.2).

So far the chemical structure of the AABP-base adducts is almost unknown. Kriek and Hengeveld [11] attempted to identify the addition products formed in liver and kidney DNA of male rats following a single intraperitoneal injection of *N*-hydroxy-*N*-4-[^{3}H]acetylaminobiphenyl. After isolation, enzymatic digestion and chromatography on LH 20 columns of the modified liver DNA they could only detect the presence of 3-(deoxyguanosine-^{2}*N*-yl)-4 acetylaminobiphenyl (25% of the bound [^{3}H]acetylaminobiphenyl residues). The remaining 75% of bound [^{3}H]AABP residues have not been identified. On the other hand, only a low amount of AABP residues bound to C_8 of guanine has been demonstrated in the case of rat liver ribosomal RNA after a single injection of *N*-OH-ABBP [12]. However, the corresponding adduct has not been detected in rat liver DNA [11].

In this preliminary report it is shown that there is both acetylation and biphenylation of DNA after reaction with *N*-AcO-AABP. More detailed accounts will be published on the reactivity pattern of DNA after reaction with *N*-AcO-AABP.

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